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Multilayered specification of the T-cell lineage fate

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Summary

T-cell development from stem cells has provided a highly accessible and detailed view of the regulatory processes that can go into the choice of a cell fate in a postembryonic, stem cell-based system. But, it has been a view from the outside. The problems in understanding the regulatory basis for this lineage choice begin with the fact that too many transcription factors are needed to provide crucial input: without any one of them, T-cell development fails. Furthermore, almost all the factors known to provide crucial functions during the climax of T-lineage commitment itself are also vital for earlier functions that establish the pool of multilineage precursors that would normally feed into the T-cell specification process. When the regulatory genes that encode them are mutated, the confounding effects on earlier stages make it difficult to dissect T-cell specification genetically. Yet both the positive and the negative regulatory events involved in the choice of a T-cell fate are actually a mosaic of distinct functions. New evidence has emerged recently that finally provides a way to separate the major components that fit together to drive this process. Here, we review insights into T-cell specification and commitment that emerge from a combination of molecular, cellular, and systems biology approaches. The results reveal the regulatory structure underlying this lineage decision.

Keywords

hematopoietic progenitor cells; T cells; transcription factors; cell differentiation; gene regulation; lineage commitment/specification

Emergence of T-cell identity: marking the pathway

T cells emerge from a microenvironmentally guided process of specialization from multipotent hematopoietic progenitors. A range of partially restricted types of immature hematopoietic precursors can respond to the microenvironment of the thymus by entering the T-cell pathway and acquiring a T-cell identity (1), and an even broader range of precursors can reveal this potential if they are experimentally exposed to T-lineage promoting signals *in vitro* (2). It is known that an indispensable driver of progression into T-lineage commitment is signaling resulting from interaction of Notch1 on the hematopoietic precursors with Delta-family Notch ligands on the surfaces of thymic epithelial cells. An *in vitro* system has been developed to support T-cell lineage determination and differentiation on the basis of coculture of the precursors with stromal cells that have been engineered to express Delta-like (DL) 1 or DL4 (3-5). However, this Notch input is needed throughout a sequence of stages in which the cells change in behavior, survival criteria, and gene expression, and all these changes require explanation. This article reviews current understanding of the transcriptional regulatory changes that convert a multipotent cell

without T-cell properties into an irreversibly committed T-cell precursor in which the T-cell gene expression program is fully under way.

Development of T cells concatenates several distinct phases in which the cells grow or develop particularly in response to signals that have quite different effects in other phases (Fig. 1). Initially (Phase 1 pro-T), the cells expand in the intrathymic microenvironment, proliferating in response to cytokine receptor signals and acquiring T cell characteristics in response to Notch interaction with Delta-like 4 (DL4) expressed in the intrathymic environment. Second (Phase 2 pro-T), the cells slow their proliferation and begin efficient rearrangement of T-cell receptor (TCR) β , γ , and δ genes, while losing sensitivity to cytokine receptor signals. Third, the cells either arrest and die or else undergo one of two further differentiation programs, a modestly proliferative one toward the $\gamma\delta$ T cell fate or a highly proliferative one, β -selection, which leads toward the $CD4^+ CD8^+$ stage [double positive (DP)] and an $\alpha\beta$ T-cell fate. Whereas all earlier stages depend continually on Notch signaling, both of these alternative survival pathways depend on the success of the cells at having assembled a complete TCR signaling complex: either a TCR $\gamma\delta$ or a 'pre-TCR' signaling complex that includes an intact, functional TCR β chain. With this TCR-dependent transition, the Notch signal becomes dispensable, and all further decisions will be dictated primarily by TCR signaling quality and TCR interaction with ligands in the microenvironment. For those cells that take the pathway toward the $\alpha\beta$ fate, the DP stage poises the cells for stringent additional steps of positive selection, further lineage sub-specialization, and a final gauntlet of negative selection.

T-cell identity depends on both negative and positive regulatory functions. In negative terms, the precursors need to divest themselves of the ability to give rise to non-T hematopoietic cell types. This is commitment, which is considered in detail below. In positive terms, the precursors need to acquire the ability to rearrange TCR genes. This requires the cells not only to turn on RAG1 and RAG2 recombinase genes but also to do so at a time when the right loci in chromatin are opened, so that the recombinase can be targeted to rearrange TCR rather than immunoglobulin genes (6,7). Targeting appears to depend on the creation of cell type-specific docking sites for RAG2, which are formed when site-specific transcription factor binding recruits histone methyltransferases to create local H3K4me3 marks (7). To introduce diversity into V(D)J joints in the TCR genes, the cells also transiently turn on the mutagenic DNA polymerase terminal deoxynucleotidyl transferase (encoded by *Dnnt*). The precursor cells also need to assemble the signaling complex components that will make TCR chains competent to deliver signals. This means turning on expression of proximal TCR signaling kinases, such as Lck, ZAP70, and Itk, T-cell specific adapters such as LAT, the invariant CD3 components of the TCR complex itself, i.e. CD3 γ , CD3 δ , CD3 ϵ , and CD3 ζ (CD247), and the surrogate α chain preTCR α (*Ptcra*), which will enable successfully generated TCR β chains to assemble into stable complexes. With the exception of the RAG genes, *Dnnt*, and *Ptcra*, this set of genes will remain active in most if not all T cells permanently and claim a major role as T-cell identity genes (Table 1).

T-lineage identity also includes other less clearly understood features, which also emerge during the early process of T-lineage commitment. One prominent feature of T cells, from mid-development onward, is their acute dependence on TCR signals to determine their survival, proliferation, or death. Taken in the hematopoietic context, the appearance of this requirement is curiously abrupt (Fig. 1), since both multipotent hematopoietic precursors and intrathymic T-cell precursors in precommitment stages can grow vigorously in response to cytokine (and Notch) signals, without any TCR at all. This early cytokine-supported phase appears to be particularly favored by the conditions in the most widely used *in vitro* culture system for T-cell development, so that populations in these stages are

disproportionately expanded relative to their steady-state levels *in vivo* (8-10). More importantly, this TCR-independent early expansion capability is also likely to provide a crucial physiological rheostat for T-cell developmental output *in vivo*. For example, thymic immigrants in fetal life undergo relatively few rounds of division before generating DP cells, while adult bone marrow derived precursors expand much more to generate a larger ultimate burst size of DP cells, but after a longer time. The difference primarily appears to be in the number of cell cycles traversed during the stages before TCR gene rearrangement (11-13). Yet shortly after commitment this capacity for continuous expansion is lost, so that population size as well as developmental status become strictly linked to TCR rearrangement. For example, RAG-deficient T-cell precursors *in vivo* proliferate to normal numbers up to the stage when TCR-dependent selection would occur, but not only fail to progress further but also fail to accumulate higher numbers at the blocked stage. This is not only due to limitations on the 'carrying capacity' of the thymus, for RAG-deficient postcommitment pro-T cells are limited in their growth and survival *in vitro* as well (12, A. Ross, R. A. Diamond, and E. V. R., unpublished results). New results imply that this dramatic switch in growth dependence may very well be linked mechanistically to T-lineage commitment, as described below.

Commitment steps: T-lineage identity by elimination

For a hematopoietic stem cell to choose a T-cell developmental fate, it must give up ~10 other developmental options. This does not happen all at once but rather by sequential exclusion events in progressively more restricted progenitors. The 'negative' aspects of T-lineage identity choice are among the best defined in all of hematopoiesis. Decades of elegant work from multiple laboratories, reviewed in several articles in this volume (14-17) and elsewhere (18-21), have shown that intrathymic T-cell precursors initially possess capacities to give rise to natural killer (NK) cells, dendritic cells (DC), macrophages and/or granulocytes (22-28). Many of these studies have employed single-cell cloning techniques, in which the same cells that exhibited these non-T potentials were also shown to give rise to T cells. More rarely, some T-cell developmental intermediates can also give rise to mast cells (29). Strikingly, however, B-cell potential is much more difficult to demonstrate in any thymocytes. Many cells that enter the thymus initially appear possess B-cell potential (30-34), at least in postnatal stages, but this potential appears to be extinguished soon afterwards; the first fetal immigrants may have undergone loss of B-cell potential prethymically (35,36). The ability to generate NK, DC, or myeloid cells persists much longer, but it is a conditional competence, seen only if the cells are transferred to permissive conditions. Within the thymus itself, most or all of these alternatives for the precursors are actively suppressed by the effects of signaling from the Notch pathway (30). Notch1 interaction with Delta ligands has the power to limit both NK and myeloid differentiation severely, blocking myeloid differentiation even under conditions where myeloid transcription factors are forcibly overexpressed (27,37,38), as well as completely aborting B-cell development. What reveals separate mechanisms of commitment at work is the response of pre-commitment T-cell precursors when they are transferred to culture under permissive conditions, with appropriate cytokines and in the absence of Delta-class Notch ligands. In these conditions, the continued potentials of the cells for NK and DC development manifestly emerge, whereas B-cell potential is not restored.

Recent work has yielded newly precise definition of the transition when the last of these alternative options are lost for mouse T-cell precursors. Fig. 1 summarizes the immature cell types in the mouse thymus that have measurable T-cell precursor activity at the single-cell level, i.e. pro-T cells. These are subsets of the 'double negative' (DN) (CD4⁻CD8⁻ surface TCR⁻) fraction of thymocytes. Of these, early T-cell precursors (ETPs) (c-Kit-high 'DN1' or 'CD4^{lo}' cells, CD44⁺ CD25⁻) are also highly efficient as precursors of NK and DC cells as

well as some other myeloid cell types, and many DN2 cells (c-Kit⁺ CD44⁺ CD25⁺) are as well. DN3 cells (c-Kit^{low} CD44^{low} CD25⁺) have lost these options completely and are good representatives of committed pro-T cells. However, there is also a split in the DN2 population, between cells that retain these alternative potentials and cells that are already T-lineage committed. This was first demonstrated by the Kawamoto group (39) using an Lck-GFP transgene to separate the subsets; we have subsequently shown that the same distinction could be made based on the level of endogenous c-Kit expression (23). DN2 cells with very high Kit levels ('DN2a') remain uncommitted while those that have started to downregulate Kit ('DN2b') are fully committed to a T-cell fate (Fig. 1). The timing is important, because this means that commitment occurs before the DN3a stage, when the cells substantially slow proliferation *in vivo*, upregulate RAG genes and undergo most of their TCR gene rearrangement (23,39). Progression onward from the DN2b stage does not require TCR gene rearrangement, but later progression beyond the DN3a stage depends on this completely. The precise ordering of these subsequent events is not as clear for cells branching off to take the TCR $\gamma\delta$ pathway as for cells taking the $\alpha\beta$ pathway, and this remains an important area for further investigation. Nevertheless, the DN2a progression to DN2b is the fulcrum of most T-lineage commitment, a one-way passage between what we may term 'phase 1' and 'phase 2' pro-T cells (Fig. 1).

Commitment and specification: two mechanisms or one?

The onset of T-cell gene expression is closely intertwined with the commitment process. All the T-cell identity genes (Table 1) are upregulated during the transition from ETP to DN3a, with all but ZAP70 achieving their full maximum expression by the DN3a stage. They differ in terms of whether they are already active in the ETP stage: LAT and Lck are already expressed in such cells at fairly substantial levels, whereas CD3 δ , CD3 ϵ , and Itk are virtually silent until the DN2 stages begin. Nearly all T-lineage identity genes are detectably expressed in populations of DN2a cells, but undergo further upregulation to the DN2b and DN3a stages. These relationships are seen not only in sorted subsets of pro-T cells from adult thymus but also in fetal thymocytes and in fetal liver-derived hematopoietic precursors differentiating along the T-cell pathway *in vitro*. With the recognition that DN2b cells are committed, and the possibility that some 'DN2a' cells might also be shifting to a committed state, it becomes an interesting question whether individual cells may delay activating genes like *Cd3e* and *Itk* until they become committed, thus linking the two processes even more tightly. However, within the compact *Cd3g-Cd3d-Cd3e* gene cluster, the equally T-cell specific *CD3g* gene is already activated to a significant extent even in the ETP population. Thus it seems likely that at least some positive regulation of T-cell-specific genes precedes lineage commitment.

Commitment by definition is an outcome of negative regulation. It implies that some differentiation pathway genes that were accessible for use or activation in the multipotent precursor can no longer be expressed. Furthermore, a cell is not committed until all its fate options are excluded. In the sequence of events in T-cell development, because the B-cell fate clearly becomes inaccessible before the NK and DC fates are excluded, there must be a minimum of two separate exclusion mechanisms that are brought into play. Later, a single negative regulator could conceivably be imagined to exclude both the NK fate and the DC fate, although these are so distinct from one another in regulatory terms that it would also be plausible for different 'commitment agents' to be involved.

The genes that such commitment factors repress could be rate-limiting for specific alternative paths through a variety of mechanisms. Very likely repression targets would be genes that encode the transcription factors that actively promote the alternative fates. They might also be genes that encode growth factor receptors that are needed to sustain cells in

the alternative fate: and there is some evidence to suggest that access to the right growth factor receptor signals may be even more of a limiting factor for early T-cell precursors to adopt the DC or myeloid fate than for them to adopt the NK cell fate (23,37,38,40). One additional way that a common negative regulator could work might be through effects on another, peculiarly T-cell specific, growth and survival requirement. Because Notch signaling inhibits many non-T pathways of development even in cells that still possess other options, the ability to 'see' these other options depends on the cells' ability to survive without Notch signaling. Interestingly, the transition from DN2a to DN2b is accompanied by a sharply increased dependence on Notch-Delta signals for survival (23). A factor that forced cells to die when Notch signals are removed could have the net impact of producing a T-lineage committed phenotype as the price of survival. These possibilities have at last become open to experimental test, as new light has been shed on the identities of transcription factors that are likely to play key roles in commitment.

Cell identity regulators: B vs. T-cell models

Two hematopoietic lineages provide models that make lineage commitment easy to understand. These are B lymphocytes and erythroid precursor cells. Both cell types are defined by batteries of highly cell-type-specific identity genes that are under direct positive regulation by a small number of transcription factors, which are themselves expressed specifically in that lineage. This is particularly notable for B cells, where the two most prominent regulatory factors are EBF1 and Pax5, factors that have no other known sites of expression or action among all other hematopoietic cell types (41-43). In erythroid cells, GATA-1 (aided by cofactors) plays a similar dominant role (44-46). The same factors are implicated in maintaining lineage fidelity by the repression of genes of alternative lineages and by repression of stem and progenitor-cell genes (43,47). This means that as few as two transcription factors can embody and directly link both the positive and the negative regulatory functions that define cellular identity.

T cells do not fit this model. T cells also depend on known transcription factors, some of which they use over and over from earliest developmental stages through to mature cell function (20,48,49). Some of these factors not only act as positive regulators of T-cell genes but are also expressed in a seemingly T-lineage-specific way. Of these, GATA-3 and TCF-1 (encoded by *Tcf7*) have been recognized for their lineage-specific importance since the 1990s, and their possible roles in commitment are considered below. However, as we have discussed elsewhere (50,51), these T-cell 'specific' factors are no more essential for T-cell development than half a dozen others that work across cell lineages and are equally necessary to generate other specific hematopoietic cell types: these include Myb, Runx1/CBF β , Ikaros, Gfi1, E2A and its relatives, and even the 'myeloid' factor PU.1 in a transient role. Even more problematically, gains of function of TCF-1 and GATA-3 do not promote T-cell development. Instead, forced activation of TCF-1 by stabilized β -catenin causes dedifferentiation and adoption of cross-lineage characteristics (52-54), while forced overexpression of GATA-3 causes DN2 T-cell precursors to jump completely out of the lymphoid 'track' and differentiate instead into mast cells (29). This means that the activation of these transcription factors does not in itself provide an explanation for the choice of a T-lineage identity.

The classic methods of germline and conditional knockouts of factors known to be important for T-cell gene expression have thus failed to identify a 'T-cell master regulator' that embodies both cell-type specific positive and negative regulatory functions. However, the candidate gene list has been limited. Conceivably also, in this cell type there could be a division of regulatory labor, such that the negative regulatory functions needed for commitment might be carried out by a factor different from the positive regulatory factors

that have been implicated in expression of T-cell identity genes. Therefore, to search more comprehensively for other possible candidates for T-cell-specific regulatory function, in the past few years we and others have used broad genome-wide screening and de novo cloning methods (55-59, J. Zhang, A. Mortazavi, B. Williams, I. Antoshechkin, B. J. Wold and E. V. Rothenberg, manuscript in preparation). Our own screening of these genes has been based on their patterns of gene expression as correlated with the T-lineage commitment process (58,59, J. Zhang, A. Mortazavi, B. Williams, I. Antoshechkin, B. Wold, and E. V. R., manuscript in preparation). This approach has yielded not only new candidates for potentially interesting regulatory molecules but also a richly detailed new picture of the T-lineage commitment process.

Complex regulatory dynamics of T-cell specification

T-cell specification and commitment emerge from dynamic changes in transcription factor expression that can be parsed into at least five major components (Fig. 2).

1. Some of the most dramatic changes are seen in a group of 'stem/progenitor'-associated genes that are initially expressed at high levels and then are downregulated during or immediately after commitment (23,57-59, J Zhang, manuscript in preparation). Many of these genes are known to be proto-oncogenes in certain contexts, and many are implicated directly in stem-cell proliferation or maintenance: the stem cell leukemia (SCL) (encoded by *Tal1*) and Lyl1 bHLH transcription factors (60-62); the Ets-family protooncogene *Erg* (63); *Lmo2* (64,65); *Meis1* (66,67); *Hhex* (previously, *Hex*) (68-70); and the Zn finger transcription factor *Bcl11a* (71). The multilineage progenitor and myeloid-cell factor PU.1 (encoded by *Sfpi1*), as well as the SNAG-domain Zn finger repressor *Gfi1b* used in erythromegakaryocytic and mast cell development, are also in this group. In detail, the downregulation of these genes is not strictly synchronous, as PU.1, *Gfi1b*, and probably SCL undergo downregulation slightly before Lyl1, whereas *Bcl11a* and *Erg* are turned off one stage later (23). This order may be slightly different in human T-cell development (56).
2. A few transcription factor-coding genes are strongly upregulated (10 fold to >100 fold) during commitment. The most strongly upregulated in the whole genome are the genes encoding the zinc finger factor *Bcl11b* and the HMG box factor LEB-1 (J Zhang *et al.*, manuscript in preparation). The alternative promoter isoform of the E protein relative HEB (*Tcf12*), called HEBalt, is also sharply induced. These are first turned on in the DN2a stage and strongly upregulated by DN2b, although Lef1 also rises proportionately more than *Bcl11b* after the DN2b stage (23,58,59). A second wave of factors is upregulated between DN2b and the DN3a stage, including *Ets2*, *Ets1*, and *Pou6f1* (not shown). With the sole exception of HEBalt, the expression of the factors in this group resembles a step function, with strong expression continuing through DN3a and the first TCR-dependent selection events and sustained or higher expression into the DP stage.
3. A much gentler upregulation (3-5-fold) from ETP to DN3a stage distinguishes the factors traditionally associated with T-cell specification, e.g. *GATA3*, *Runx1*, *Gfi1*, *TCF-1* (*Tcf7*), and the canonical form of HEB (*Tcf12*).
4. There is a sustained 'legacy' of stem-cell and multipotent progenitor-associated regulatory gene expression that continues throughout the T-cell specification and commitment process. Even though many of these legacy genes are turned off later, during β -selection or $\gamma\delta$ -selection, they continue to be expressed at minimally changing levels from the uncommitted ETP stage through T-lineage commitment and into the DN3a stage. *Myb*, *E2A* itself, and *Ikaros* are all strongly expressed as

well but almost unchanging at the RNA level, before, during, and after commitment. They are therefore present to contribute potential regulatory functions to the lineage commitment process but are clearly not rate limiting for commitment.

5. There is also a group of regulatory genes (not shown) whose expression peaks sharply at the DN3a stage, after commitment, and then subsides or disappears if and only if the cells undergo TCR-dependent selection. SpiB is a prominent example (58). The transient use of these genes at the DN3a stage is most likely to be associated with the specific physiological features of that stage, such as TCR gene rearrangement, $\gamma\delta$ vs. $\alpha\beta$ lineage separation, cell cycle arrest, and poisoning of cells for TCR-dependent selection, rather than with the acquisition of a T-cell identity as such.
6. After commitment, a separate group of factors (not shown), prominently including Aiolos (Ikzf3), is upregulated during the DN3a/3b transition if and only if the cells undergo TCR-dependent selection. Id3, the glucocorticoid receptor (Nr3c1), and Schnurri-3 (Hivep3), also increase moderately from ETP to DN3 stage but may play their most important roles only during TCR-dependent selection.

In light of the timing of commitment, the first and second groups of transcription factors provide the most salient candidates for regulators of T-cell identity, negative and positive regulators, respectively. Of the first group, SCL and PU.1 have been most closely examined for effects on T-cell development if their expression is artificially sustained at a high level. Both of these block T-cell development and promote T-cell leukemia under these conditions (37,60,72-75). In addition, sustained expression of PU.1 has the power to divert even post-commitment DN3 cells to develop into myeloid and dendritic cells, in accord with known PU.1 roles in these alternative pathways (37,38,73,74). In spite of these strong inhibitory effects, however, even massive overexpression of PU.1 has no lasting inhibitory effects on T-cell development provided that it is transient (37,73). As PU.1 is also required for development or expansion of competent T-cell precursors (76,77), and as Lyl1 is also essential to sustain lymphoid precursor potential in stem cells (62), the early expression of these factors may be crucial for the self-renewal capacity of precursors during the important intrathymic phase before commitment. In physiological conditions these factors may provide a regulated restraint on the timing of progression to a T-lineage committed state (78).

The regulatory genes that undergo upregulation in the second group are the most provocative source of candidates for drivers of T-lineage commitment. This is a small group, however, and its members have some surprising features. Most of them are not T-lineage specific. Ets1, Ets2, and Pou6f1 are all expressed in B cells, NK cells, and/or myeloid cells as well as in T cells (58,79,80, <http://www.immgen.org>). Lef1, although not highly expressed in mature B cells, is highly activated during B-cell development, where its knockout may have a stronger effect than on T-cell development (81). This leaves Bcl11b as the one transcription factor gene that appears to be turned on exclusively during T-lineage commitment, which is also T-lineage specific in its expression and sustained throughout T-cell maturation and function. Bcl11b is crucial for $\alpha\beta$ lineage T-cell development, and a profound defect has been reported at β -selection when Bcl11b is absent (82,83). Yet curiously, although TCR β rearrangement is inhibited in Bcl11b-knockout pro-T cells *in vivo*, RAG gene expression is fully induced in the absence of Bcl11b (84). In a developmental context, there is little evidence for Bcl11b acting as a positive regulator of T-lineage identity genes in general (84). Thus, the one T-cell specific factor that sits at the cusp of the phase 1/phase 2 transition may not work by turning on T-lineage-specific genes. Nevertheless, it provides a crucial function for T-lineage developmental progression as well as commitment.

Bcl11b functions at the commitment checkpoint

Bcl11b is a zinc finger transcription factor with a DNA binding domain of six zinc fingers and a partially characterized N-terminal repression domain (85). It is unique among all important factors that are involved in early T-cell development because of its sharp onset of mRNA expression in the DN2a stage. In mouse thymocytes, the four exons of the *Bcl11b* genetic locus are spliced into four identified mRNAs (86, Li and Rothenberg, unpublished data). Full length Bcl11b cDNA encodes a protein of 884 amino acid with six C2H2 zinc fingers, but its level in developing T cells is relatively low; instead, the mRNA containing exons 1, 2, and 4 (Bcl11b 1-2-4) is the abundant isoform. Bcl11b 1-4 is an isoform which is specifically implicated in γ -irradiation induced thymic lymphomas (86) and may also be generated as a result of an intragenic deletion caused by illegitimate RAG recombination activity (87). Because all six zinc fingers are located in exon 4, Bcl11b 1-2-4 and Bcl11b 1-4 have the same zinc finger domains as the full length four-exon form. Biochemical studies have shown this factor interacts with Sirt-type HDACs or NuRD to repress gene transcription through trichostatin-resistant and trichostatin-sensitive mechanisms (85,88). Many of its known roles are repressive (85,89-91), though not all (92,93). In early T-cell development, we have found that Bcl11b indeed acts to repress genes associated with stemness and alternative lineage potentials in T-cell precursors, although it remains to be determined whether it does this directly (84).

If Bcl11b is deleted acutely from otherwise normal precursors just before the initiation of T-cell development, it has three effects on T-cell specification (84,94,95) (Fig. 3). First, it blocks progression of the cells in the T-cell pathway (84,95). Second, Bcl11b loss prevents the cells from losing access to a cluster of myeloid fates (84,95). Third, deletion of Bcl11b greatly increases access of the cells to an NK fate alternative (94, see reviews by P. Liu and J. Di Santo in this volume). Induction of the NK-specific transcription factor Zfp105 is a particularly sensitive indicator for the loss of Bcl11b in pro-T cells (Fig. 4). NK development also becomes less susceptible to inhibition by Notch signaling when Bcl11b is absent, and a peculiar class of Kit⁺ CD11c^{+/low} NK-like cells with exuberant growth properties can emerge (84,94). These defects closely correspond to failures in the complex of events that normally occur during the phase 1 to phase 2 transition (Fig. 1).

In vivo, Bcl11b-deficient thymocytes are arrested with a CD25⁺ CD44^{low} phenotype that suggests arrest just prior to β -selection (83). These thymus populations include some TCR $\gamma\delta$ cells, consistent with RAG recombinase function and the ability to signal through the TCR complex (83,89). Although TCR β rearrangement appears to be reduced, the β -selection developmental block cannot be fully rescued by a TCR β transgene (82), suggesting that Bcl11b deficiency causes a more profound defect as well. As shown by conditional deletion at a later stage of development, Bcl11b is important for T-cell survival and activation (90,96). However, at this early stage, the primary effect of Bcl11b is not required for survival. *In vitro*, with growth driven by DL1 and a rich cocktail of cytokines, loss of Bcl11b instead causes T-cell precursors to become blocked in a self-renewing, Kit^{high} DN2a state under developmental conditions that should fully support T-lineage progression (84,95) (Fig. 3, 'self-renewal', Fig. 4A, B).

The comparison between *in vivo* and *in vitro* phenotypes of Bcl11b-deficient DN cells suggests some inconsistency in the developmental arrest points (82-84,89,95), but this may be more apparent than real. One difference is that the IL-7, Flt3, Kit ligand, and Notch ligand levels *in vitro* may permit DN2 cells to expand more than in the thymic microenvironment, where IL-7 signals at least are probably limited (97). Bcl11b-deficient cell proliferation and differentiation are exquisitely sensitive to the levels of these environmental signals (84). In addition, the Bcl11b-deficient cells can adopt an ambiguous

phenotype in which they can downregulate CD44 to levels typical of DN3 cells while Kit levels remain high, as typical of DN2 cells. Although little is known about how Kit and CD44 are regulated in DN stages, their levels usually appear to correlate very well to each other and to the progression of T-cell development. However, even *in vivo*, Bcl11b-deficient cells in the thymus can persist for many weeks with a Kit⁺CD25⁺CD44^{low} phenotype. Fig. 5 shows populations of donor-type cells from the thymus of a reconstituted radiation chimera, in which retroviral transduction of Cre had been used to delete Bcl11b and activate a ROSA26R-YFP reporter in the bone marrow donor cells prior to adoptive transfer. By 10-16 weeks after engraftment, most YFP⁺ cells from donors with a wildtype Bcl11b genotype and carriers had become either CD4 or CD8 positive, while some adopted a CD44⁺ but Kit^{low} CD25⁻ phenotype. In contrast, YFP⁺ Bcl11b-deficient donors consistently gave rise to a much larger population of DN thymocytes, and these were distinguished by their uniformly higher expression of Kit. Many were Kit⁺ CD44⁺ CD25⁻ cells, which also expressed NK cell markers (not shown), resembling the NK-like cells that are generated *in vitro* ['DN1-like' in (84)]. In addition, however, Bcl11b-deleted donor cells could generate a pro-T-cell population which combined the Kit^{high} CD25⁺ phenotype of DN2a cells with the low CD44 level of DN3 cells (Fig. 5C, arrows). Crucially, the expansion of this cohort of cells *in vivo* without further developmental progression suggests another difference from normal DN3 cells, a functional equivalent of the DN2a stage at which Bcl11b-deficient cells accumulate *in vitro*.

The pivotal DN2 stages: Notch, growth factor response, and Bcl11b

The DN2a stage when Bcl11b is first turned on is the first step unambiguously recognizable along the T-cell-specific developmental pathway. The characteristic induction of CD25 surface expression at this stage is profoundly dependent on Notch-Delta signaling and on other rate-limiting conditions that remain less well defined (98,99). These cells already express nearly maximal levels of the T-cell-specific factors GATA-3 and TCF-1 as well as nearly maximal levels of the Notch and E protein target gene Hes1 (23). Notch signaling is needed to initiate Bcl11b expression (59), and P. Li *et al.* (94) have found physical interactions of CSL (RBPJ κ) at least two *cis*-regulatory sites in the *Bcl11b* locus where Notch complexes may work directly toward turning on this gene. Normally *in vivo*, transit through this stage is probably fast, as few cells accumulate as DN2a in steady state before moving forward through commitment. However, the DN2a state can also be actively sustained for many rounds of cell division by cytokine-dependent growth *in vitro*, and so this population has discrete characteristics of its own that are not just artifacts of a mixture of progenitor and T-lineage committed cells. Most importantly, normal DN2a cells still strongly transcribe the full array of progenitor-associated genes (group 1) at levels similar to those in ETPs. Therefore, the Notch signals that turn on CD25 and activate GATA-3 and TCF-1 and initiate Bcl11b induction are not in themselves sufficient to dictate the repression of the progenitor cell genes.

Notch signals in themselves are also not sufficient to impose T-lineage commitment. This has been proven elegantly by Ikawa *et al.* (95) using a minimalistic tissue culture system for cell-free presentation of DL4 to developing precursors. In this system, multipotent hematopoietic progenitors reach the DN2a stage efficiently and proliferate well to the cytokines that probably sustain growth *in vivo*, i.e. Kit ligand, Flt3 ligand, and IL-7. However, they cannot become committed under these conditions and cannot progress further in the T-lineage pathway. The progression and commitment defects can be linked to their failure to activate Bcl11b expression under these conditions (95). In the system used by Ikawa *et al.* (95), Bcl11b induction depends on stepping down the intensity of signals from the IL-7 receptor: at lower IL-7 concentrations the cells can progress. As we have shown using a different experimental strategy, the progression in response to lower IL-7 depends

directly on Bcl11b, since Bcl11b-deleted cells remain stuck in DN2a, even when the IL-7 concentration is reduced (84).

If Bcl11b is absent, the complex of specification events that normally accompanies the DN2 stage is split. Several distinct tiers of regulatory events show markedly different effects (Fig. 6). Most of the T-cell transcription factors that are induced or preserved as a legacy from stem and progenitor cells continue to be expressed normally. This includes the upregulation of GATA-3, TCF-1, HEB, Gfi1, and Notch targets like Hes-1, the sustained expression of Myb, Runx1, Ikaros, and E2A, and even the sharper upregulation of HEBalt. The upregulation of RAG-1, which normally occurs after Bcl11b induction, is also Bcl11b-independent. Furthermore, a group of T-cell identity genes including *Cd3e*, *Cd3g*, and *Ptcra* are not affected by Bcl11b deletion in their initial upregulation. But as these genes should increase expression normally after the DN2a stage, they fail to be upregulated further in Bcl11b-deficient pro-T cells (84,95). Finally, many of the T-cell genes that normally would be turned on during the DN2b stage, such as *Id3*, *Zap70*, and *Ets2*, remain at substantially reduced levels of expression. Thus, Bcl11b may not be an essential driver for the initiation of specification, but it is required for the completion of specification. Bcl11b is probably both a downstream target and a mediator of many effects of Notch signaling in early T-cell development: while progression from ETP to DN2a stage depends on Notch signaling, the progression from a DN2a regulatory state to a DN2b regulatory state depends on Bcl11b.

Dissection of mechanisms of commitment via Bcl11b

Commitment is correlated with the downregulation of stem/progenitor genes, and in pro-T cells, this downregulation depends on Bcl11b. It is not the only factor that contributes to T-lineage commitment, as discussed below. However, the behavioral changes in Bcl11b-deficient cells are tightly linked to the status of stem/progenitor-associated gene expression. This is a strong result because, unlike most other good candidates for T-lineage commitment factors, Bcl11b is not required for survival or proliferation of DN2a stage precursors; Bcl11b-deficient cells even appear to have a selective advantage *in vitro* and can be readily characterized. When Bcl11b deletion causes the cells to be trapped in a self-renewing DN2a state, even after weeks of growth with Notch-Delta signaling they maintain high expression of the full set of stem/progenitor regulatory genes (84)(Fig. 6).

PU.1 provides one example of how the stem/progenitor genes can themselves create access to the regulatory states promoting alternative pathways. The mechanisms through which PU.1 is silenced in DN2b-DN3a stage T cells appear to depend on Runx1 and possibly TCF-1 (75,100,101). How Bcl11b may promote this repression is not yet clear. Still, PU.1 is not just a stem/progenitor factor but also a direct driver of the myeloid and dendritic cell gene expression programs, so that PU.1 repression can be causally connected with loss of these options. In Bcl11b-deficient DN2 cells, the mechanisms that contribute to PU.1 repression are not deployed, and these pro-T cells become an abnormally efficient source of Mac-1⁺ Gr-1⁺ myeloid cells and dendritic cells (84,95).

Bcl11b complements the effects of Notch signaling in restraining myeloid and dendritic cell potential. Notch signaling can overcome even forced high-level PU.1 expression to preserve T-lineage identity (37,38,102), and presumably antagonizes myeloid and dendritic fates even before Bcl11b is turned on. Although they continuously express high levels of PU.1 and multiple progenitor-cell associated factors, Bcl11b-deficient DN2a cells still need to be removed from Notch-Delta signals and supplied with myeloid cytokines, such as M-CSF (CSF-1), in order to undergo myeloid differentiation (Fig. 6). Therefore, Bcl11b is not required for the mechanism used by Notch to constrain these fates. It may be that Bcl11b's role *in vivo* is primarily to promote one-way exit from the stages in which PU.1 expression

keeps the myeloid option available. Nevertheless, the power of Notch to protect cells from myeloid diversion by PU.1 increases sharply at the stages when Bcl11b is induced (37), and it is possible that Bcl11b participates directly in a mechanism to block myeloid differentiation (M. M. Del Real, L. Li, and E. V. R., unpublished results).

A different mechanism must be invoked to explain how the NK cell option might be related to Bcl11b roles in the normal T-cell pathway. Greatly increased access to the NK cell program is among the most dramatic effects of Bcl11b deletion (94). Unlike the myeloid options, this diversion is fully supported by the same cytokine environment that would otherwise support T-cell development, and it can be seen even while Notch signaling is present, although it is enhanced by reduced access to Notch-Delta interaction (84;94). Bcl11b-deficient cells upregulate NK-lineage transcription factors Zfp105 (84;94), Eomesodermin, Zbtb16 (PLZF), and Nfil3 (E4bp4), even before full differentiation to NK-like phenotype (84)(Fig. 6). However, in contrast to the stem/progenitor genes, these NK-promoting genes are not part of the normal gene expression program of the ETP and DN2a stages. Thus, before Bcl11b is normally induced, these genes can be kept silent by other mechanisms. But are these genes really alien to the T-cell program? Fetal pro-T cells are biased to generate specialized lineages of $\gamma\delta$ T cells, and such $\gamma\delta$ T cells do express high levels of Id2, IL-7 α , and IL-2/IL-15R β , a subset of genes which also support the intrathymic pathway of NK cell development (103). When Bcl11b deletion in fetally derived precursors causes Id2, Il7ra, and Il2rb genes to be further activated (84), this could occur by channeling the cells' regulatory state toward one of the available T-lineage differentiation programs. However, there remains a question of where the positive signal comes from that turns the NK-promoting transcription factor genes on. Zbtb16 and Nfil3 are not known to have any normal roles in ETPs, and Zfp105 and Eomes are normally silent from ETP through DN3 stages in these precursors (80, <http://www.immgen.org>, J Zhang, *et al.*, manuscript in preparation). Thus, it is likely that Bcl11b loss may indirectly lead to introduction of additional signals, for example via IL-2/IL-15R β signaling, that supply the positive regulatory inputs that activate *de novo* expression of Zfp105 and Eomes.

One highly attractive possibility is that Bcl11b indirectly blocks NK differentiation by promoting the activity of E proteins, which can provide a critical barrier to the NK cell pathway (104). There is no obvious effect on RNA levels encoding E2A or HEB (Tcf12) expression themselves when Bcl11b is deleted (84). However, E protein effects on T-cell development are determined not only by their own expression but also by the levels of antagonistic or diversionary heterodimerization partners, including both the Id proteins that antagonize DNA binding altogether and the SCL/Lyl1 subgroup of bHLH factors that redeploy them to a distinct set of gene targets. When Bcl11b is deleted, there is concerted upregulation of at least three genes that could collectively act as E protein antagonists or decoys, including Id2, Tal1 (SCL), and Lyl1. If SCL and Lyl1 collaborate with Id2 to antagonize E protein activity in this context, this could provide a link between ongoing stem/progenitor cell gene expression and access to the NK pathway. As E protein activity also appears to limit myeloid development (105), this mechanism could even preserve the access of Bcl11b-deficient cells to myeloid fates as well.

Thus, loss of Bcl11b positively endows cells with three different options that would normally be foreclosed by the DN2b stage: enhanced NK cell differentiation even when some Notch signaling is present; enhanced myeloid differentiation provided that Notch signaling is removed; and the option to continue to grow as a poised DN2a cell without further developmental progression (Fig. 3). Blocked E protein activity could contribute to at least two of these effects, although other regulatory abnormalities need to be evaluated as well. It is important to distinguish the options that Bcl11b loss does not provide. Bcl11b-deficient pro-T cells have not yet shown any ability to transdifferentiate into B cells. It

follows that the lineage restriction factors that block B-cell development must remain intact in these cells. Additional hematopoietic fates, nominally more ‘distant’, may also remain excluded: these must be tested in future work. Without Bcl11b, however, cells cannot cross the divide from ‘phase 1’ pro-T-cell status to ‘phase 2’ (Figs 1 and 6).

Commitment effectors besides Bcl11b: multiple contributions from broadly expressed T-lineage transcription factors

Bcl11b does not act alone in T-lineage commitment downstream of Notch, although its role greatly clarifies the overall biphasic aspect of the process. Recall that positive regulation of T-cell identity genes begins normally in the absence of Bcl11b (Fig. 6). It follows that most essential positive regulators of T-lineage genes can be mobilized in a Bcl11b-independent way. Importantly, additional lines of evidence implicate several of these factors, Runx1, TCF-1, GATA-3, and E proteins, in specific aspects of commitment as well. All of these regulatory factors are normally expressed *in vivo* at substantial levels both before and after the onset of Bcl11b expression. None appear to be altered in expression by loss of Bcl11b (84), and only E protein activities, as already noted, would be predicted to be affected by Bcl11b deletion. Therefore, the roles of the others should provide inputs to the developmental status of pro-T cells that are distinct and independent from Bcl11b.

Runx/CBF β compound factor activities are required for at least two aspects of early T-cell specification and commitment. First, a CBF β dose-reduction strategy has been useful to observe specific early T-lineage effects mediated through any of the three Runx family genes while bypassing the severe hematopoietic effects of full Runx1 deletion. This system has shown that Runx/CBF β activity is acutely required for generation of true DN2 stage pro-T cells (106). Interestingly, some cells can upregulate CD25 in response to Notch/Delta signals even when CBF β dosage is extremely low, but these ‘DN2-like’ cells fail to turn on Bcl11b or CD3 ϵ expression, and are distinct even from normal early DN2a cells because of their lack of GATA-3 expression (106). Thus, Runx/CBF β is probably required to work upstream of both Bcl11b and GATA-3, at the very earliest point in T-cell specification. A second clear role for Runx/CBF β complexes is during commitment, to execute downregulation of PU.1. Runx factors bind to three sites in the major upstream regulatory region for PU.1 and thus act directly (100,107). The repressive activity in pro-T cells has been demonstrated *in vivo* (100) as well as in a cell culture system where high-dose Runx1 has been found to mediate pro-T-cell specific repression of PU.1 through an additional, functionally dedicated repression *cis*-regulatory element (101). It is still unclear how this repressive action of Runx factors at the *Sfp1* locus is timed, since expression of Runx1 and other Runx factors is already high during phase 1 pro-T-cell development as well as after commitment. One possibility is that the shifting balance between Runx1 and the other two Runx factors, Runx2 and Runx 3, is important (58). Alternatively Runx factors could enter complexes with additional, T lineage-specific factors to trigger nucleation of repression complexes at the DN2b stage.

TCF family factors are tempting to consider as T-lineage commitment factors. These factors are bifunctional, with activating roles in the presence of nuclear β -catenin and repressive roles where β -catenin is not recruited. While still somewhat controversial, the best established role for TCF-1 and Lef1 is during β -selection, long after commitment (108-111), and this is also the stage when most effects of experimental manipulation of TCF/ β -catenin interaction are seen (112-115). However, perturbations of this axis can also cause a severe disruption of T-cell development from an early stage (116,117). Disruption of the TCF-1 coding gene (*Tcf7*) has a much more severe and consistent effect than perturbation of the β - or γ -catenin partners. This suggests that a major role of TCF-1 in the earlier stages may normally be to act as a repressor. Currently, one target that this factor is suggested to repress

is PU.1 itself, through a site associated with the major upstream regulatory region (75); however, the effect appears to be a relatively subtle, modulating one. However, as for Runx factors, TCF-1 expression is extremely high before as well as after T-lineage commitment, suggesting that it is not rate limiting for this change. Meanwhile, the close TCF-1 relative Lef1 does increase expression dramatically during T-lineage commitment, but it has even higher expression in pro-B cells, so this feature is not T-cell specific. An unresolved question is whether the dramatic upregulation of Lef1, beginning only slightly later than Bcl11b, might alter the net function of TCF/Lef family members in T-cell precursors so as to cause repressive effects to become dominant in the DN2b stage. However, the limited available evidence suggests that TCF-1 and Lef1 work redundantly (118) and primarily support growth-promoting functions in early T cells (119) as well as in pro-B cells (81).

GATA-3 has been implicated in T-cell specific gene expression (120-122) and has been known to be essential for T-cell development for over 15 years (49,123-126). However, its full role has proved difficult to resolve because of its intense dose-dependent effects. Although cells with reduced GATA-3 expression can acquire a DN2-like phenotype, there are severe viability effects at both ETP stages and afterwards (127). Conversely, overexpression of GATA-3 can be as antagonistic to T-cell development as it is to most developmental alternatives (128,129). GATA-3 is probably involved in two aspects of T-lineage commitment, working through different mechanisms. Forced GATA-3 expression is intensely inhibitory for B-cell development (29,129, D. D. Scripture-Adams, A. Arias, K. J. Elihu, A. Champhekar, M. Zarnegar, & E. V. R., manuscript submitted), consistent with the hypothesis that the early upregulation of GATA-3 in the ETP stage may be directly linked to the loss of B-cell potential. Unpublished data cited elsewhere in this volume (16) strongly support this role. At a later stage, GATA-3 may also play a direct role in excluding myeloid and dendritic cell fates (130), by repressing PU.1 transcription (29), or by antagonizing any residual PU.1 protein activity as the GATA-3:PU.1 ratio shifts in the DN2a-DN2b transition. GATA-3 has also been implicated in restricting access to the plasmacytoid dendritic cell fate in human cells by antagonizing the PU.1 relative, SpiB (130). Bcl11b deletion leaves GATA-3 expression unchanged or slightly enhanced, however, and so GATA-3 is particularly likely to contribute to B-lineage exclusion and any other aspects of commitment that do not depend on Bcl11b.

While other factors may be suggested to exclude B and myeloid fates, the only pro-T cell factors besides Bcl11b that are known thus far to antagonize the NK cell developmental alternative are the E proteins, in T-lineage cells E2A and HEB (*Tcf12*) primarily (104). The molecular pathway through which these factors limit NK development is not yet clear. It is possible that the NK antagonism consists simply of promoting T-lineage progression at the expense of the NK fate. Similarly, in prethymic precursors loss of E protein favors myeloid development, suggesting a role for E proteins in antagonizing this option as well (105,131). But E proteins are also particularly interesting because of their strong contribution to T-cell gene expression, specifically to expression of the set of genes that characterize the immediate postcommitment DN3 state. Many T-cell identity genes appear to be regulated directly by E2A, especially in combination with Notch signaling (132,133). Furthermore, E protein activity appears to be required to maintain the one-way nature of the DN2 to DN3 progression. When both E2A and HEB are conditionally deleted at the DN3 stage, the cells reportedly regain the ability to proliferate strongly in response to IL-7, and they revert to an apparent DN2-like phenotype (134). Whether or not these cells fully regain expanded developmental potential has not yet been determined. Nevertheless, this report implicates net E protein activity in another of the same roles as Bcl11b, including enforcement of the directionality of progression from phase 1 to phase 2 pro-T-cell development.

Unleashing the expression of T-cell identity by negative regulation?

The central unresolved question of T-cell specification continues to be the identity of the particular combination of positive regulators that accounts for the surge in expression of T-cell identity genes between the DN2 and DN3 stages. The positive regulators that are known to participate, as briefly reviewed in the previous section, are already strongly expressed by the ETP stage and only modestly altered in their expression afterwards. Massive discontinuities in gene expression, such as those for the CD3 cluster and Bcl11b, seem unsatisfying to explain as linear responses to the ~3 fold increase in GATA-3 or TCF-1 or the 3-5-fold increase in HEB (Tcf12). One may consider three general solutions. First, there could be an unknown rate-limiting positive factor involved, although the genome-wide analysis is rapidly narrowing the possibilities for this. Second, activation of T-cell gene expression could be multiplicative, dependent on coincident inputs from all of the modestly upregulated T-cell factors. There are indeed *cis*-regulatory elements that can carry out this kind of combinatorial ‘and’ logic with independent inputs (135,136), and the prediction would simply be that all the positive factors would need to converge on *cis*-elements of this type. However, a third possibility is also worth considering, as the data do show some manifestly dramatic changes in transcription factor expression during T-lineage commitment that go far beyond 2-5 fold effects. These changes are (i) the downregulation of multiple stem/progenitor-associated regulatory genes and (ii) the upregulation of one factor, Bcl11b, that is most likely to act as a repressor. Thus, the third possibility is that these negative regulatory events could themselves result in the positive regulation of T-cell identity genes.

Formally, this possibility is like the paradigmatic ‘double negative gate’ that operates a crucial cell fate decision in the sea urchin embryo (137,138). In the classic double negative gate mechanism, the positive inputs for the regulated genes must all be present in advance, but all the targets are initially kept silent by a common repressor. Then if a second-tier repressor is introduced to silence the first, the target gene battery can be explosively activated, exclusively where the second-tier repressor is present. Signatures of this mechanism are the Boolean, non-gradual form of the split between the states of target genes fully repressed in one stage (or territory of the embryo), and fully unleashed in another. There are notable aspects of early T-cell specification that appear to fit this model: for example, the fact that most or all known positive regulatory inputs are active in the cells by the ETP stage, before the cells undergo specification, and the fact that upregulation of the most specific T-cell identity genes is accompanied predominantly by silencing of stem/progenitor-cell regulatory genes. But, could Bcl11b be the secondary repressor that relieves repression for all these targets?

The answer depends on how the stem/progenitor regulatory genes are working in early T-cell development. There are two aspects of the question. The first is whether mechanisms can be invoked that would enable these factors to be the ‘first-tier repressors’, i.e. to interfere with T-cell gene activation. The second is whether such a role makes sense in view of the sustained expression of these stem-cell genes, through many cell divisions and days of Notch-Delta signaling, up through the DN2a stage.

Multiple mechanisms do in fact exist that would cause factors in the stem/progenitor group to block activity of the known T-cell positive regulators on their targets. Already noted in this review is the ability of SCL (Tal1) and Lyl1 to inhibit the activation of T-cell-specific target genes by E proteins (60,139). A second mechanism is the ability of high-level PU.1 to block activity of GATA-3 or *vice versa*, through protein/protein interaction, depending on which one is in excess. This mechanism is an extension of the widely discussed ‘switch’ that emerges from mutual antagonism of PU.1 and GATA-1 (140,141). The PU.1/GATA-3 version has recently been shown to operate in rare peripheral T-cell subsets in which PU.1

expression is reawakened (142,143). A fourth gene to be sharply downregulated at the DN2a/2b boundary, Gfi1b, also has several potential antagonistic roles, one as a repressor of GATA-3 (144) and one as a repressor of its own relative, Gfi1, which is also required for T-cell specification. Thus, as long as the stem/progenitor group factors are expressed, they collectively limit the activity of E proteins, GATA-3, and Gfi1 all together, not only by restraining transcription but also by blocking the activity of whatever positive regulatory protein is present. This effect could indirectly stall the activation of a broad range of 'DN3' genes, and in fact these are the results seen when PU.1 levels are artificially locked at a high level in pro-T cells (78). All these antagonisms could then be relieved at a stroke by whatever Bcl11b-dependent mechanism is used to silence this set of stem/progenitor genes.

This proposal thus raises the question of why the T-cell specification program could tolerate expression of the stem-progenitor genes so long if the goal of the program is truly to promote T-cell differentiation. The answer here may be to recollect the split between phase 1 and phase 2 (Fig. 1), both of which are integral to pro-T cell development. Precursor expansion is a profound, system-level requirement in any developmental scheme that incorporates selection as stringent as the TCR-based β -selection, positive selection, and negative selection that T-cell precursors will later undergo. Phase 1, the cytokine-supported expansion of precursors before TCR gene rearrangement, is crucial for the establishment of a normal T-cell precursor pool. Not only does it make sense to have this phase capitalize on the use of stem/progenitor self-renewal functions; it also makes sense to force a regulated delay before the differentiation of the cells to phase 2, when their individual reckonings with fate may no longer be postponed.

Conclusions

In this review, we propose that T-cell specification occurs in two distinct phases, defined by the overlap between several distinct 'layers' of regulatory factors. One important layer is the stem/progenitor cell set that promotes cytokine-dependent self-renewal functions, but may antagonize full differentiation. These factors become overlaid by a second group that includes the early-acting T-cell factors such as GATA-3 and TCF-1 and inputs from the Notch pathway. At some point, the convergence of these regulatory inputs and the attenuation of IL-7 receptor signaling signal the activation of a crucial transitional factor, Bcl11b, which quickly sets in train a mechanism to silence the stem/progenitor self-renewal genes. One result is the extinction of the last alternative lineage potentials. Simultaneously, this clears the way for unleashing full activity of the T-cell factors and their stably expressed collaborators, such as E proteins, both by transcriptional and protein-level mechanisms. This newly dominant constellation of factors then works through phase 2 of pro-T-cell development to establish the definitive T-cell state. This state makes the cells subject to mandatory TCR-dependent selection and may have a countdown aspect; it no longer permits open-ended expansion by the previous, TCR-independent mechanism.

There are many links in this chain that require experimental test and validation, as well as biochemical mechanisms that must be resolved in order to explain the action of the factors in the functions proposed. The direct targets of Bcl11b need to be identified and the structures of both activation and repression complexes for T-cell specification need to be established. The regulatory inputs into Bcl11b itself, as well as the T-cell specific *cis*-regulatory elements for *Bcl11b*, *Lef1*, and the *Cd3* genes, all need to be identified. However, this framework for considering early T-cell development may provide some order in the complexity that enables a rational regulatory structure to be discerned.

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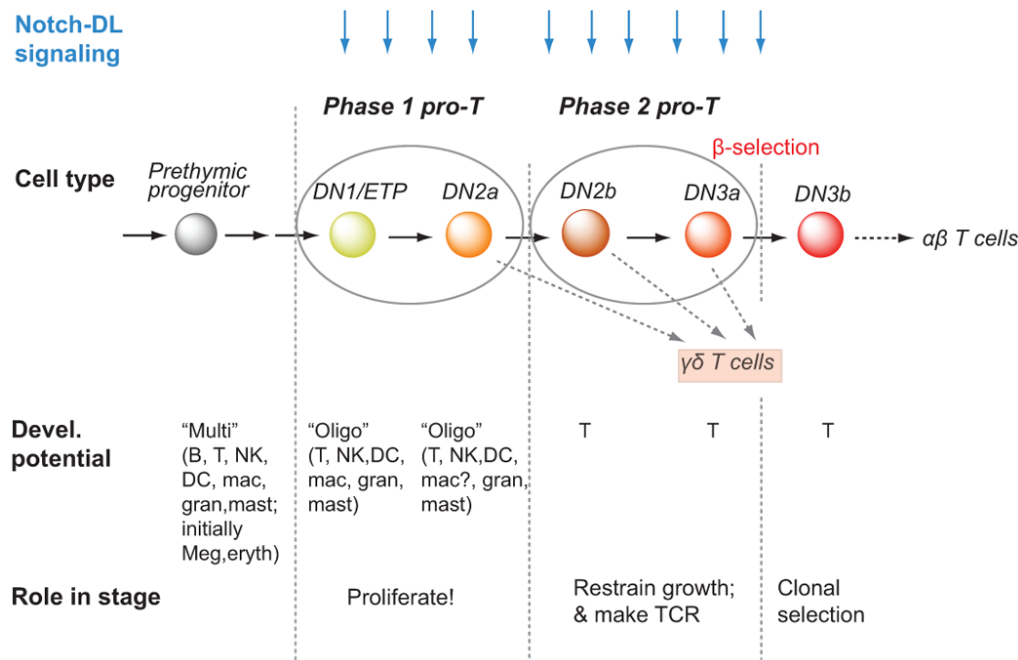


Fig. 1. Stages in T-cell specification and commitment

Double negative ($CD4^-CD8^-$ surface TCR^-) stages between thymic entry and the first TCR-dependent selection events are highlighted. Signals from Notch-Delta interaction are required throughout both 'phase 1' and 'phase 2'. Proliferation is strongest in phase 1 and in cells that successfully rearrange $TCR\beta$ genes, after β -selection. $\gamma\delta$ cells have a somewhat different program that is not discussed in this review. Mac, macrophage; gran, granulocyte; Meg, megakaryocyte; eryth, erythrocyte. The DN1 cells that have actual T-cell precursor activity are Kit^{High} , i.e. ETPs.

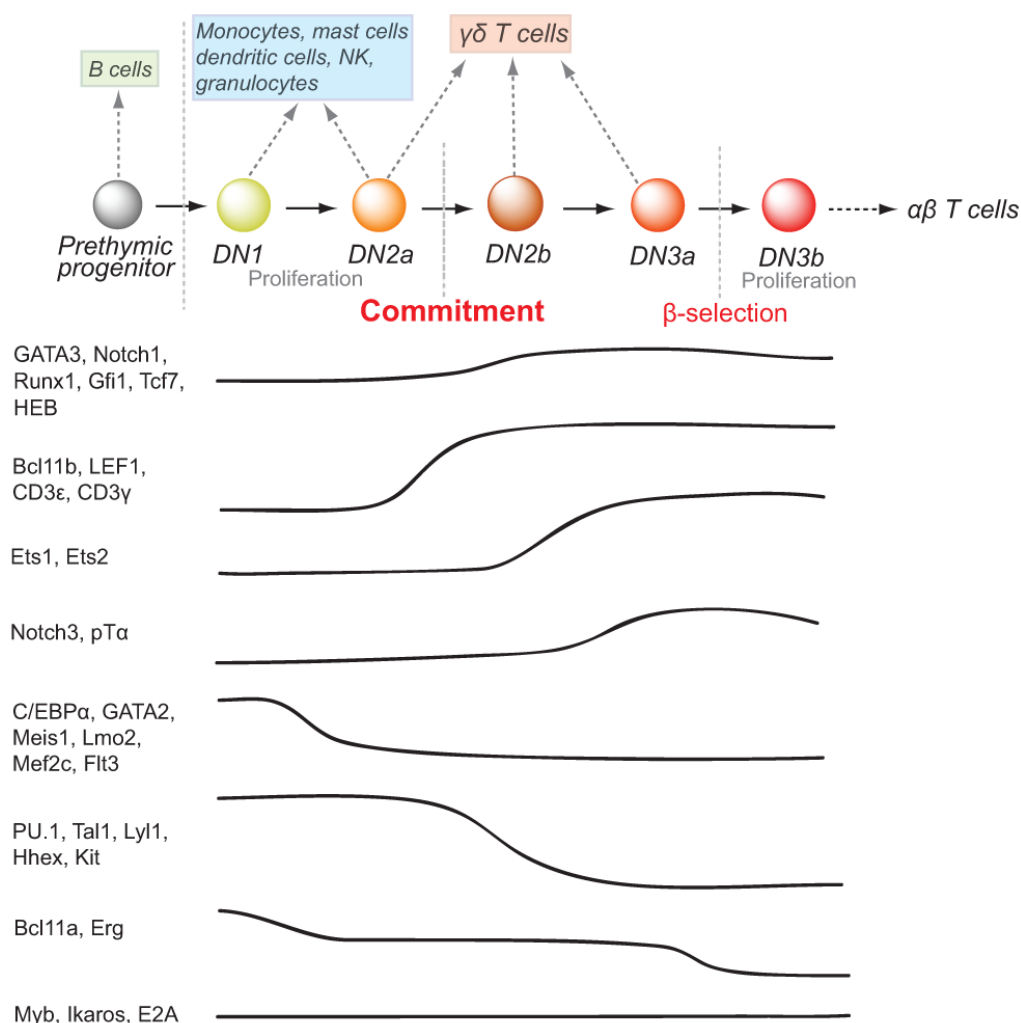


Fig. 2. Regulatory gene expression changes during T-cell specification

Curves show approximate timing of increases and decreases in expression of the indicated regulatory genes and key T-cell identity genes, selected from the groups discussed in the text. Data are from ref. (58) and J. Zhang *et al.*, manuscript in preparation. RNA transcripts encoding Myb, Ikaros, and E2A (bottom) are expressed at high but relatively stable levels throughout the indicated stages.

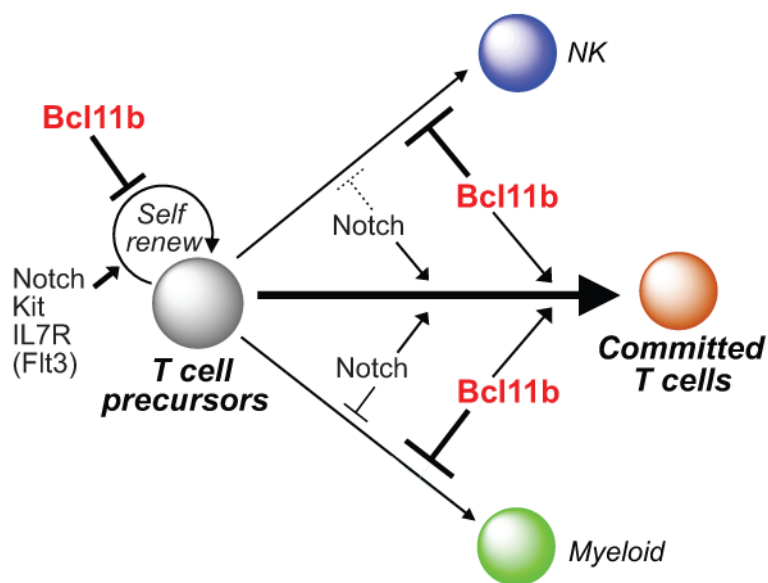


Fig. 3. Effects of Bcl11b deletion on pro-T cells: increased self renewal, prolonged access to myeloid and dendritic fates, and enhanced, long-term access to NK cell fates
 Bcl11b is not required for Notch signals to inhibit myeloid differentiation, but Bcl11b deletion weakens the ability of Notch signals to inhibit NK differentiation. Gene regulatory alterations proposed to underlie these shifts are shown in Fig. 6.

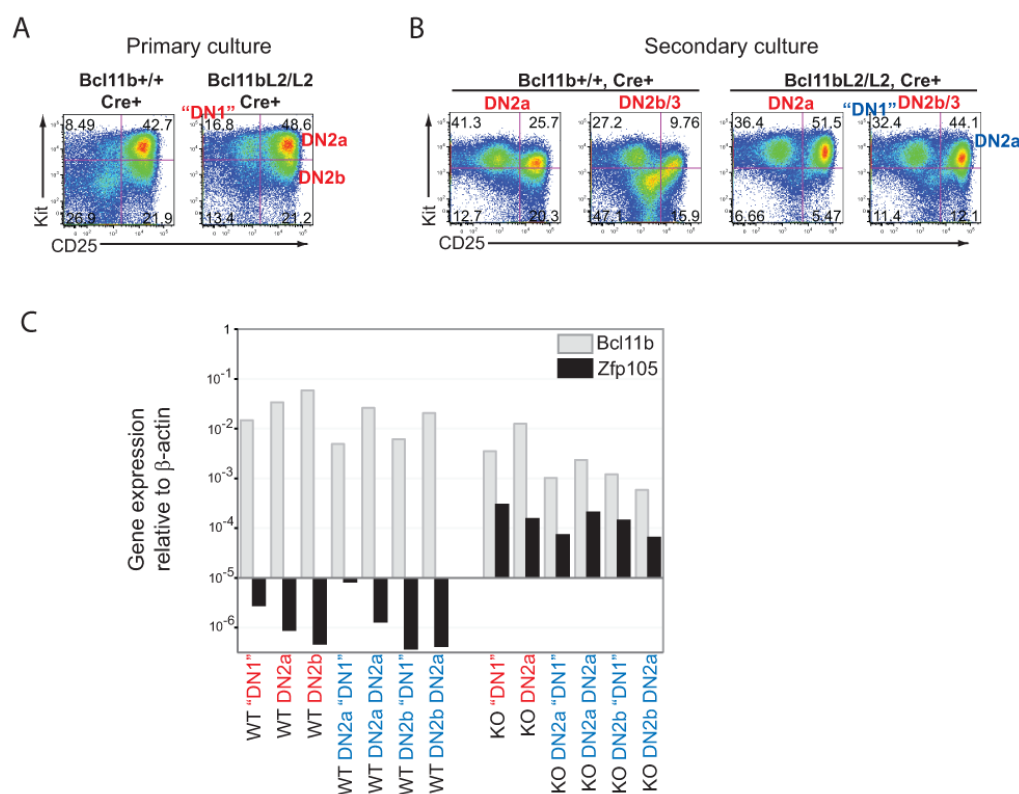


Fig. 4. Enhanced upregulation of NK-cell specific transcription factor Zfp105 in pro-T cell populations with reduced Bcl11b gene dosage

This experiment shows efficient upregulation of this normally silent gene even when experimental deletion of Bcl11b is incomplete. (A) Primary cell populations derived from fetal liver, differentiating in T-cell conditions with OP9-DL1 stroma) for a week after treatment with retroviral Cre. Left and right panels show control and Bcl11b-deleted precursors, respectively (from ref. 84). Positions of populations sorted for gene expression and returned to secondary culture are labeled in red. (B) Secondary cultures derived from the populations shown in (A), after 12 days of further OP9-DL1 culture. Populations sorted for further RNA analysis from each culture came from gated regions labeled in blue. (C) Real-time PCR analysis of gene expression in the populations derived from primary and secondary cultures in A and B, from control (WT) and Bcl11b-deleted (KO) samples. Measurement of spliced transcripts of Bcl11b, as a measure of exon 4 deletion efficiency, and of the NK-specific transcription factor Zfp105. Note the selection for cells with reduced levels of intact Bcl11b in the secondary cultures of Bcl11b-deleted cells (KO, compare red-labeled and blue-labeled populations) and the marked upregulation of Zfp105.

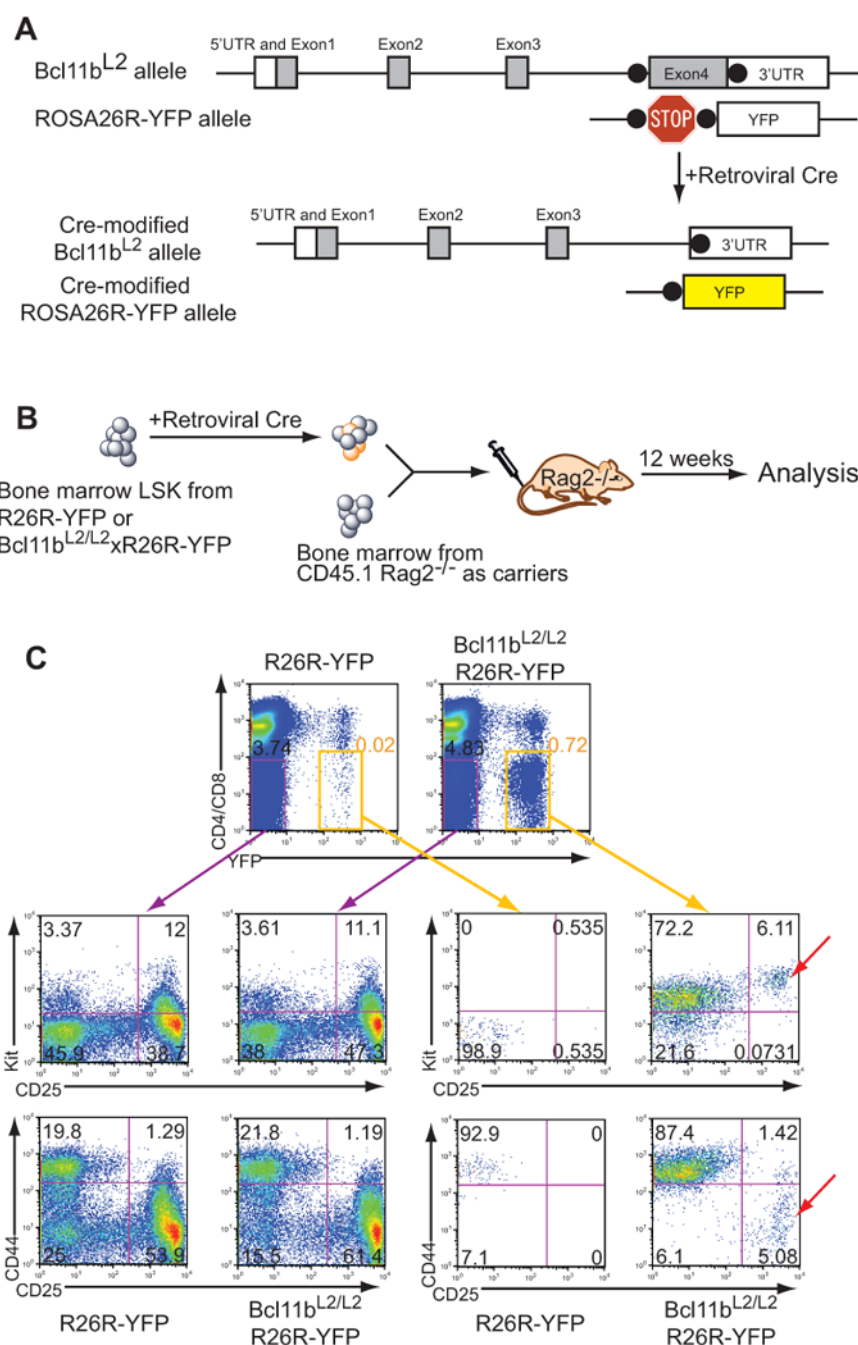


Fig. 5. Fate of Bcl11b-deleted bone marrow cells in the thymus of radiation chimeric host after adoptive transfer

Donor cells were Lin⁻ bone marrow cells from Bcl11b-floxed and Bcl11b wildtype mice, each with a Cre-dependent ROSA26-YFP marker to monitor successful Cre exposure. Cells were treated with retroviral Cre *in vitro* and then used to reconstitute irradiated hosts. (A) Diagram of donor alleles. (B) Experimental scheme: in this version of the experiment, Rag-deficient hosts were used. (C) Phenotype of donor-type YFP⁺ cells in the thymus of recipient mice. Note that the Cre treatment marks a cohort of cells that tend not to persist in the thymus normally after 12 weeks (R26R-YFP only, right panels). However, the loss of Bcl11b (Bcl11b^{L2/L2} R26R-YFP) consistently increases the accumulation of donor cells in

the thymus and the retention of Kit on the surfaces of NK-like and pro-T cell-like (arrow) CD4⁻CD8⁻ populations.

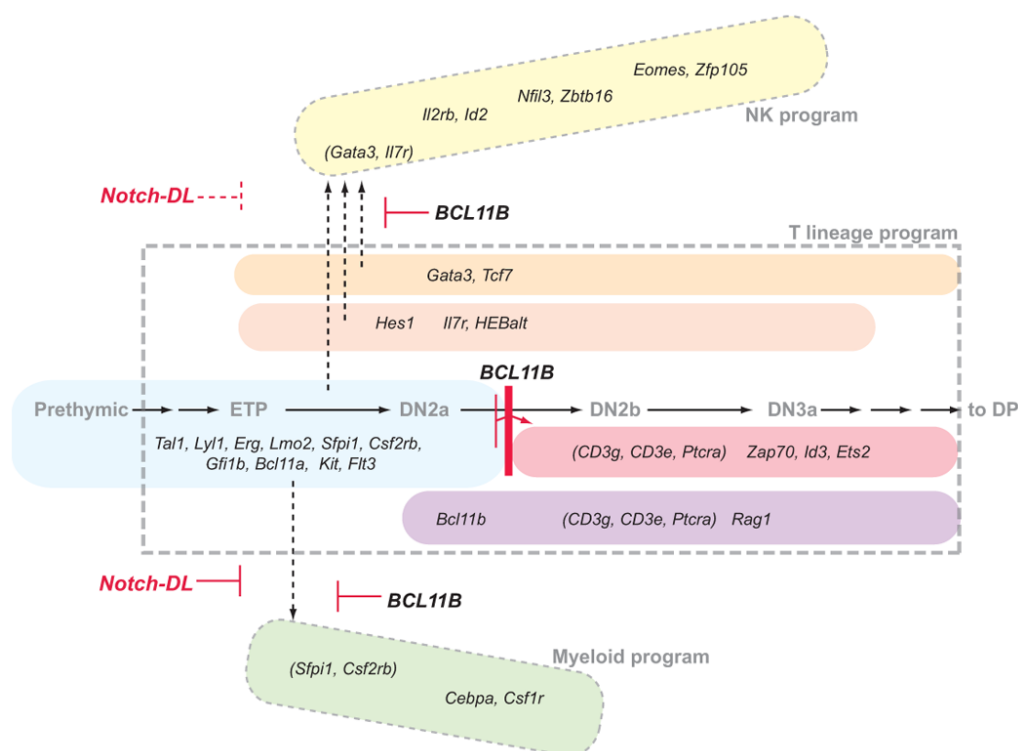


Fig. 6. Separate regulatory subroutines in pro-T-cell commitment: selective effects of loss of Bcl11b in the phase 1 to phase 2 transition

The figure relates the functional effects shown in Fig. 3 to the gene expression patterns shown in Fig. 2 and their alteration in Bcl11b-deficient pro-T cells. Groups of genes that interact in coordinated segments of the T-cell specification process are depicted in 'layers', as well as groups of genes that are activated specifically when the cells escape the T-cell program to enter NK or myeloid/dendritic cell programs. Genes shared between two regulatory states are in parentheses. *Cd3g*, *Cde3*, and *Ptcra* are upregulated in two steps, where the first (violet layer) but not the second (pink layer) can occur without Bcl11b. Note that Bcl11b locus activation does not depend on Bcl11b function (84). Not shown: whereas CD3 and Bcl11b gene activation is sustained in later T-cell development, some genes induced in the DN2 stage (*Ptcra*, *Rag1*) are downregulated at β -selection.

Table 1
Molecular definitions of T-cell identity

T-cell 'identity genes'	Genes with transient roles in TCR assembly
<i>Lck</i>	<i>Ptcr</i> α (PreTCR α)
<i>Zap70</i>	<i>Rag1</i> , <i>Rag2</i>
<i>Itk</i>	<i>Dn</i> <i>tt</i> (Terminal deoxynucleotidyl transferase)
<i>Lat</i>	
<i>Cd3g</i> (CD3 γ)	
<i>Cd3d</i> (CD3 δ)	
<i>Cd3e</i> (CD3 ϵ)	
<i>Cd247</i> (CD3 ζ , CD247)	